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ABSTRACT

Preferential metastasis of prostate cancer (PCa) to bone marrow contributes to disease mortality and morbidity. Local factors present in the bone stromal extracellular matrix (ECM) increase metastatic tumor growth through paracrine interactions among tumor, stromal and endothelial cells present in the microenvironment. Heparin binding growth factors (HBGF) present in bone marrow provide growth and survival signals to metastatic PCa cells. Perlecan (Pln), the major heparan sulfate proteoglycan (HSPG) in the bone marrow stromal ECM functions as a co-receptor to deliver HBGFs to high affinity receptors. HSchains on Pln are located in a unique N-terminal domain I (PlnDI) which serves as a co-receptor for HBGFs. We hypothesized that Pln, specifically PlnDI, delivers HBGFs to the PCa cell surface, promotes cell survival and protects from apoptosis. We used three PCa cell lines: LNCaP, C4-2B, and PC3 to study the function of PlnDI. We harvested conditioned medium (CM) from bone stromal cell lines, HS27a and HS5, which produce abundant Pln. The proteoglycan rich fraction containing Pln was obtained using anion exchange bead chromatography, then tested as a survival factor for the three different PCacell lines. DNA fragmentation experiments showed that bone stromal derived Pln from CM protected all three lines of PCa cells from camptothecin induced apoptosis. We also supplied purified, fully glycosylated recombinant PlnDI protein to PCa cells, which also protected cells from apoptosis. Pln knockdown clones grow poorly compared to the parental or control transfected C4-2B cells (Ref.1).Interestingly, knockdown cells weremore susceptible to apoptosis in response to the apoptosis inducers anti-Fas and camptothecin, and exogenous PlnDI rescued survival.We followed this up by undertaking to identify natural factor(s) produced by bone marrow stromal cells that induces apoptosis in PCa cells. We found that this apoptosis inducing activity is specific for LNCaP derived lines. Ongoing experiments seek to identify this factor.Collectively, our studies demonstrate that Pln present in the bone marrow compartment protects PCa cells from apoptosis and that this activity maps to HS bearing PlnDI.This function contributes to the growth and survival of PCa cells in a potentially hostile bone marrow microenvironment.

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Introduction

Metastasis of prostate cancer cells to bone marrow stroma occurs with high fidelity. The paracrine interactions between prostate cancer cells and surrounding bone stromal cells are key to disease progression [3]. A number of heparin binding (HB) growth factors including bFGF (FGF-2), VEGF and hepatocyte growth factor/scatter factor (HGF/SF) stimulate growth of prostate cancer cells [4-7]. In addition to affecting proliferation, HB growth factors promote cell motility, stimulate angiogenesis and inhibit apoptosis, all of which contribute to cancer progression [8-10]. HB growth factor receptors depend on heparan sulfate proteoglycans (HSPGs) as co-receptors for signaling [11]. Pln is an abundant HSPG found in developing and adult animals in various species [13]. It also is a major HSPG in the bone stromal ECM [14,] and blood vessels including that of the prostate that can support high-affinity receptor binding. Pln protein core is composed of 5 major domains with a unique domain I and 4 other domains with sequence similarities to other ECM and cell surface proteins [17]. Three potential glycosaminoglycan (GAG) attachment sites occur near the amino terminus. In most cases, the GAGs are of the heparan sulfate (HS) variety, although chondroitin sulfate (CS) may sometimes substitute for HS at one or more sites [20]. The large, complex nature of Pln suggests that this HSPG interacts with multiple cell surface and ECM proteins, only some of which have been described [22]. Several growth factors as well as other soluble proteins of the cell surface and ECM bind to the HS chains of Pln in domain I [23], and perhaps even to the Pln core protein [24]. Therefore, by binding various growth factors and interacting with cell surface HB growth factor receptors, Pln may mediate a number of biological actions previously associated with these growth factors and heparin (Hp)/HS polysaccharides.

Functions of individual Pln domains have been studied. Pln domain I (PlnDI) is a unique module at the N terminus and contains three closely spaced Ser-Gly-Asp sequences that serve in GAG attachment [26]. PlnDI with HS chains binds a number of matrix molecules, cytokines, and growth factors [28]. Our previous studies indicate that the GAG-bearing PlnDI provides a sufficient signal to trigger C3H10T1/2 cells to enter a chondrogenic differentiation pathway and the GAGs are important for this process because PlnDI-based polypeptides lacking GAG chains either by enzymatic removal or mutation of HS/CS attachment sites were inactive [29]. This growth factor delivery "co-receptor" function of PlnDI was explored with regard to prostate cancer bone metastasis and growth.

During our PlnDI study, we found that exposing LNCaP and C4-2B cells to conditioned medium (CM) harvested from bone stromal cell lines HS27a/HS5 initially induced apoptosis in PCa cells. This indicated that there are likely to be hostile factors in the bone microenvironment that inhibit PCa cell growth, and that overcoming the actions of these factors is a key element in metastatic PCa progression.

The first phase of this training period focused on the role of PlnDI in cell proliferation, cell survival and apoptosis by using different prostate cell lines and Pln knockdown clones and various biological assays. The second training phase extended the study to

characterize natural paracrine factor(s) produced by bone marrow stromal cells that induced apoptosis in PCa cells.

Main Body

Specific Aim1: To test the co-receptor function of PlnDI in prostate cancer cell proliferation.

Preliminary experiments tested the HB growth factor dependent responses of Pln knockdown clones and compared them with their parental cell line C4-2B or control transfectants [1]. We used a structure-function based approach to determine if PlnDI, specifically the GAG chains and/or the core protein in domain I, are sufficient to provide this co-receptor function. Surprisingly, although Pln knockdown clones have lower proliferation rate, adding purified PlnDI in the medium didn't rescue the phenotype. Since the intact PlnDI didn't rescue the reduced proliferation, we didn't precede the GAG chain study (Fig. 1). Our hypothesis is that PlnDI only is not sufficient for the co-receptor function involved in proliferation.

Specific Aim2: To test if PlnDI is involved in activating the cell survival pathway.

We measured activation of the cell survival pathway Akt and MAPK by Western blot and compared responses of the Pln knockdown clones and the parental C4-2B cell line. Although the proliferation rate of the knockdown clones was different, there were no detectable changes in Akt or MAPK pathways of these knockdown clones (Fig. 2), thus we discontinued the study the role of PlnDI involved in these pathways. The key factors in these pathways are highly expressed in these PCa cells. That could explain why we could not detect moderate changes due to Pln knockdown. Another hypothesis is that Pln regulated proliferation goes through other pathways besides Akt/MAPK.

Specific Aim3: To test if PlnDI protects prostate cancer cells from apoptosis.

To study the role of PlnDI involved in apoptosis, we expanded the study to three PCa cell lines: LNCaP, PC3 and C4-2B. We used two bone stromal cell lines HS27a and HS5 to harvest conditioned medium. The proteoglycan rich fraction containing Pln was obtained using anion exchange bead chromatography. Including the Pln enriched medium with all three PCa cell lines protected from apoptosis compared to serum free medium after treatment with apoptosis inducer, camphothecin (Fig. 3). This experiment indicated that the Pln in the bone environment might protect prostate cancer cells from apoptosis. We also applied purified PlnDI protein to these cells and successfully rescued the cells from apoptosis (Fig. 4). Next, we measured the ability of PlnDI added exogenously to Pln knockdown clones to protect them from apoptosis induced by anti-Fas or campthothecin. We showed that PlnDI can rescue the increased apoptosis caused by Pln knockdown (Fig. 5). These data suggest that Pln can protect prostate cancer cell from apoptosis and PlnDI is sufficient for this function. We plan to expand our study by using Pln antibody depletion to ensure the role of Pln in cell apoptosis.

Specific Aim4: To identify natural factor(s) produced by bone marrow stromal cells that induce apoptosis in PCa cells.

We first co-cultured LNCaP and C4-2B cells with bone stromal cell line HS27a/HS5. This configuration allows cells in different compartment to exchange soluble factors without cell-cell contact. Surprisingly, LNCaP and C4-2B cells did not show increased cell growth in the co-culture condition. On the contrary, the number of cells decreased after 3 days co-cultured with mixture of HS27 and HS5 cells (Fig. 6). The similar effect were seen when culture these cells in conditioned medium (CM) harvested from HS27a/HS5 cells. The cell proliferation dropped dramatically after 3 days (Fig. 7). These results suggest that the bone stromal cell conditioned environment has an inhibitory effect on PCa cell growth. The CM harvested from bone stromal cells not only inhibited PCa cell growth, but also induced apoptosis (Fig. 8A). Neither of the other two PCa cell lines PC3 nor DU145 showed any apoptosis after treatment of CM. None of the other cell lines tested showed any apoptosis (Fig. 8B). Breast cancer cell line, T47D, did not show apoptosis after treatment of CM suggesting that the apoptotic effect of CM is preferential to PCa cell lines. Bone osteoblast cell line, MG63, and prostate epithelial cell line, PrEC, did not show apoptosis in response to CM suggesting that the CM is not targeting normal bone and prostate cells. These data suggest that LNCaP and its sublines are regulated through unique cell survival and apoptotic pathways that are not present in all cells derived either from bone or prostate. The CM from prostate foreskin cell lines or prostate stromal cells failed to induce apoptosis in the LNCaP and C4-2B cells (Fig. 9). This indicates that the prostate microenvironment is not hostile for prostate cancer cells. This CM induction of apoptosis was dose dependent (Fig. 10). We showed that the LNCaP cells and C4-2B cells showed 10% more cell death compared to those grown in SF medium (Fig. 11). When grown in CM harvested from bone stromal cells, the surviving LNCaP and C4-2B cells altered their cell morphology (Fig. 12). The cells lost the typical epithelial cell phenotype and normal cell-cell contact. Cells were elongated to spindle shape and formed long processes. This phenotypic change indicates that these cells are undergoing a phenotypic transformational.

In figure 13, we propose a new model of paracrine interaction between prostate cancer cells and bone stromal cells. Unlike previously models, we propose that after the cross talk of bone stromal cells and prostate cancer cells, the paracrine factors from bone stromal cells induce apoptosis of some PCa cells. Surviving cells undergo a series of gene expression and morphology changes, further adapting to growth in the bone microenvironment. Based on the data that PlnDI can protect prostate cells from apoptosis, we believe that Pln acts as a local survival factor to help PCa cells as they further adapt to factors present in the bone environment.

Figures

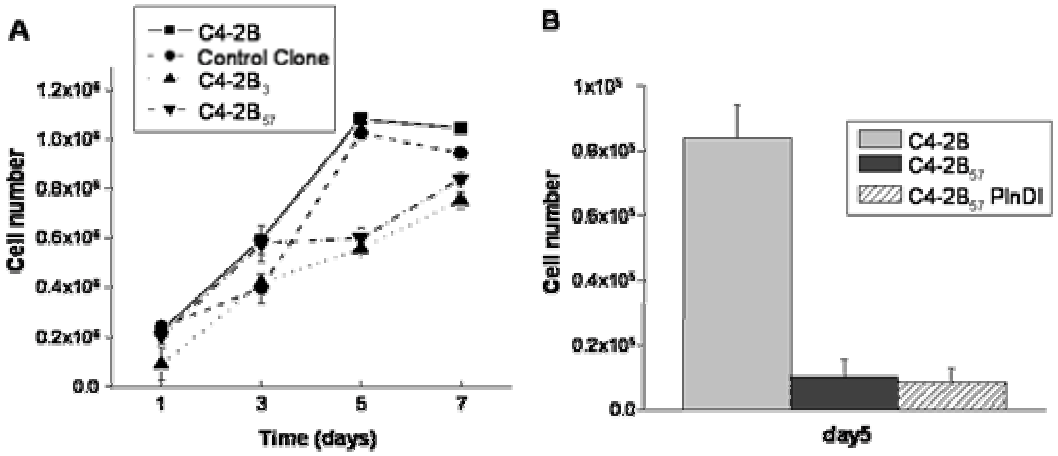

Figure 1. Addition of exogenous Pln does not rescue growth responses to FGF-2 in Pln knockdown C4-2B cells. Panel (A). Conditioned medium from parental C4-2B cells failed to rescue the proliferation of Pln knockdown clones C4-2B$_3$ and C4-2B$_{57}$ cultured in FGF-2. Panel (B). Purified Pln domain I protein (5 ug/ml) failed to rescue the growth response to FGF-2, whereas parental cells continued to proliferate through the fifth day in culture. Figure is adapted from [1].

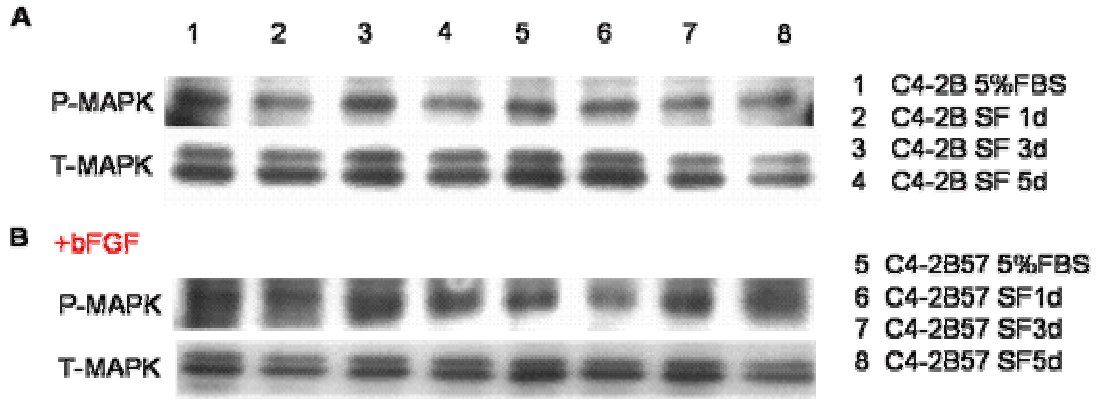

Figure 2. Western blots showed that Phosphorylated MAPK level was not altered in the Pln knockdown clone 57. P-MAPK: phosphorylated MAPK. T-MAPK: Total MAPK.

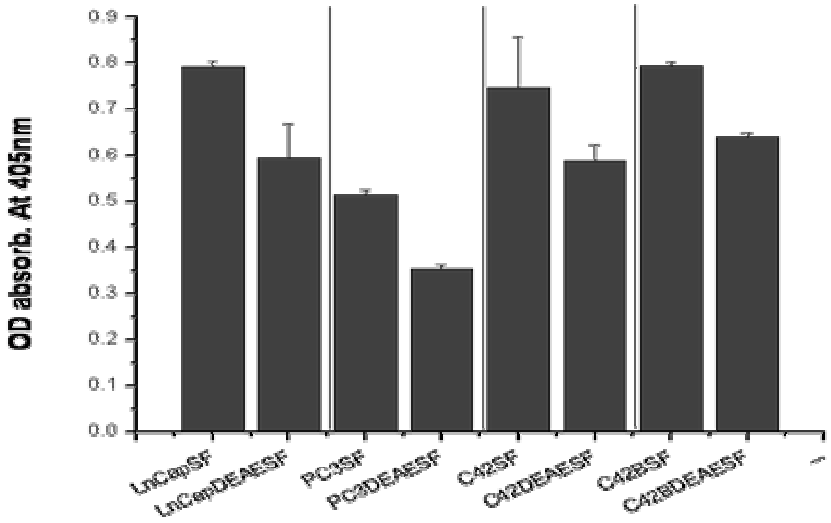

Figure 3. Pln enriched medium reduced apoptosis in all cell lines treated with camptothecin.

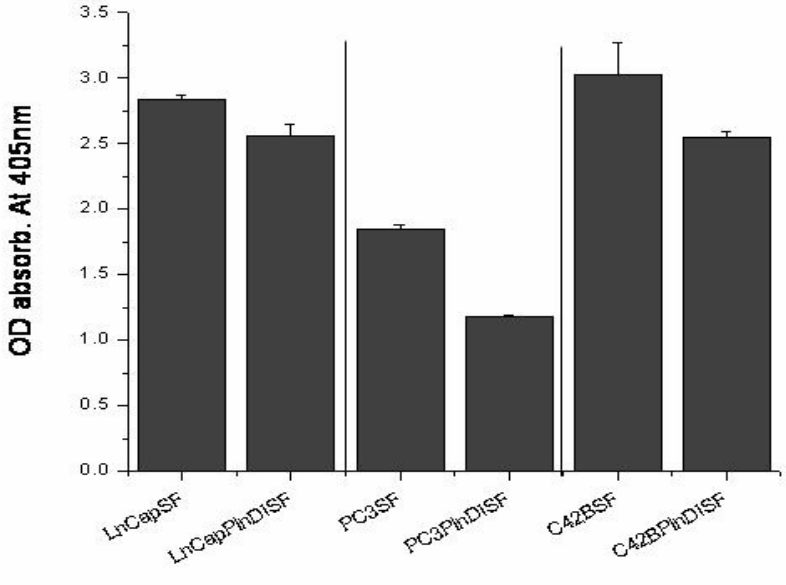

Figure 4. PlnDI reduce apoptosis in all cell lines treated with camptothecin.

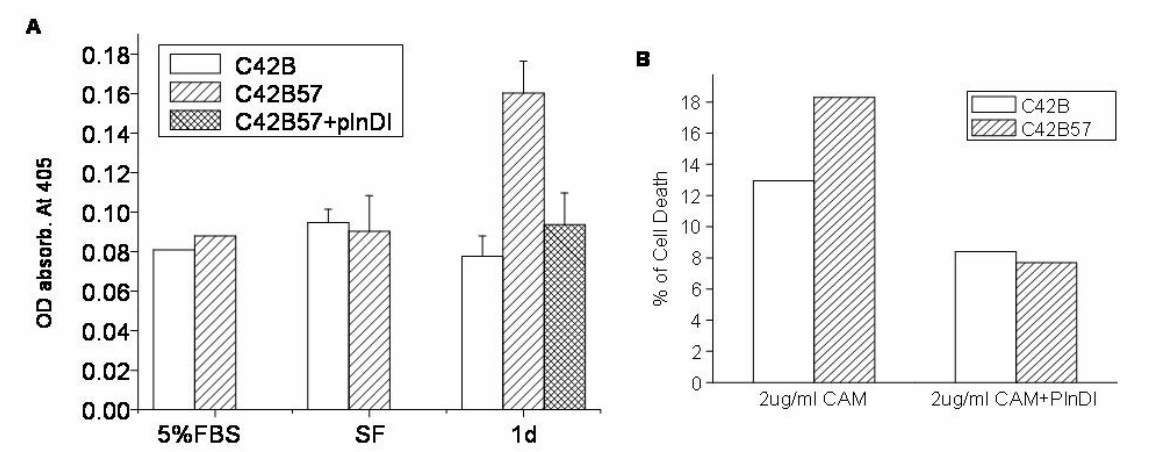

Figure 5. Pln knockdown clone underwent more apoptosis compared to parental cells, and PlnDI can rescue apoptosis induced by Anti-Fas or camptothecin. Panel (A). DNA fragmentation assay showed that Pln knockdown clone underwent more cell death in response to Anti-Fas treatment. Panel (B). Pln knockdown clone had more cell death in response to camptothecin.

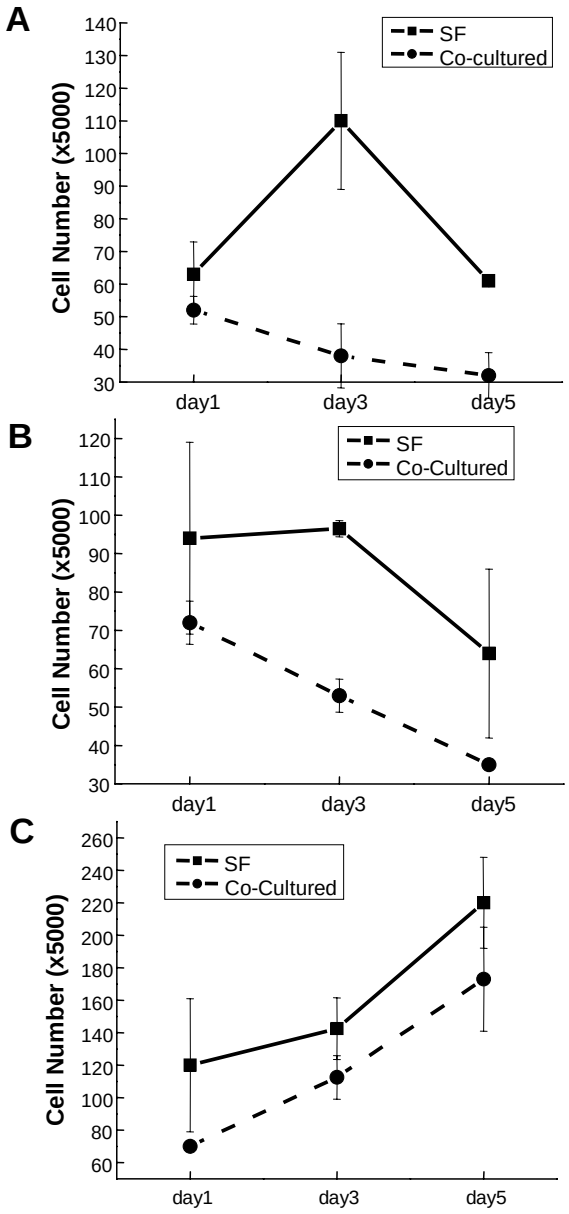

Figure 6. PCa cell lines LNCaP (A) and C4-2B (B) had less growth when co-cultured with bone stromal cell lines compared to SF medium. PC3 (C) had the same growth rate compare to SF medium. Cells were counted after co-culture with bone stromal cell lines HS27a/HS5 for 1, 3 or 5 days.

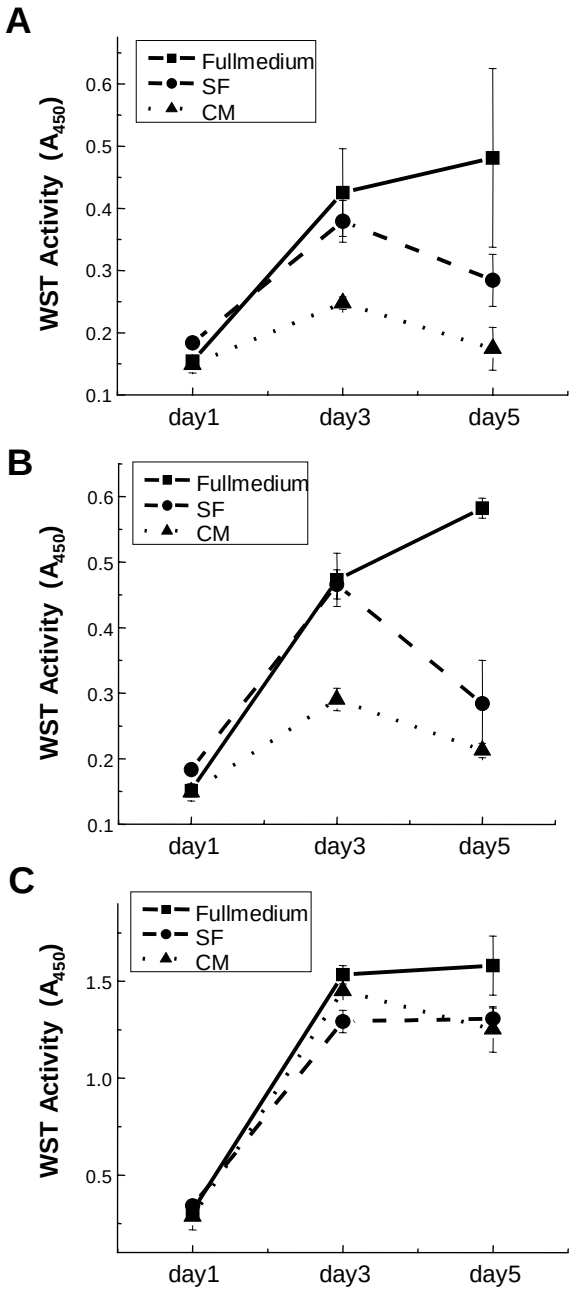

Figure 7. Conditioned medium (CM) reduced LNCaP (A) and C4-2B (B) cell proliferation but not PC3 (C) cell proliferation. Cells were seeded in 96 well plates. Cells were treated with full medium (10% (v/v) FBS), serum free medium or conditioned medium. Medium were changed every other day. WST solution were added in the medium for 15 minutes at days 1, 3 and 5, and then absorbance read at OD 450nm.

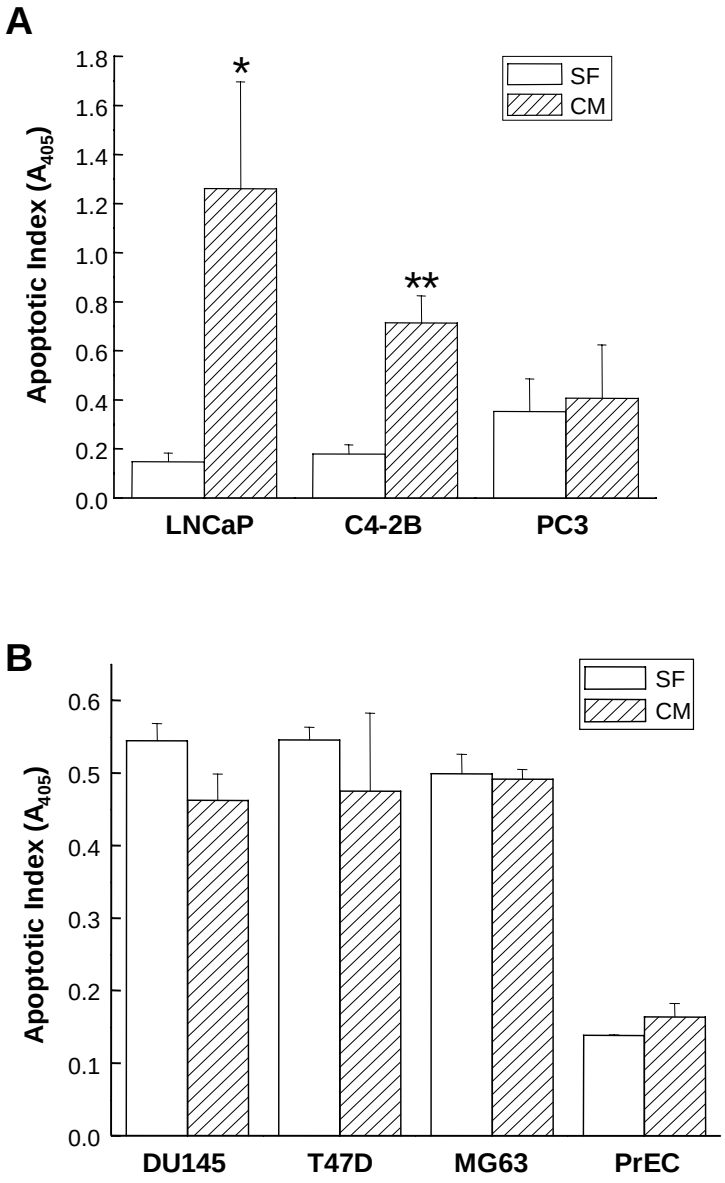

Figure 8. Conditioned medium (CM) harvested from bone stromal cell lines initiated apoptosis in LNCaP cells and C4-2B cells, but not PC3 cells (A) or selected other cell lines (B). Cells were seeded in 24 well plates and then treated with serum free (SF) medium or CM for 2 days. Apoptosis was measured using DNA fragmentation ELISA. For different experiments, the basal level of OD 405nm varied due to the limitation of the method, so only apoptosis caused by CM were compared to corresponding SF treated cells. (*$p<0.05$, ** $p<0.005$)

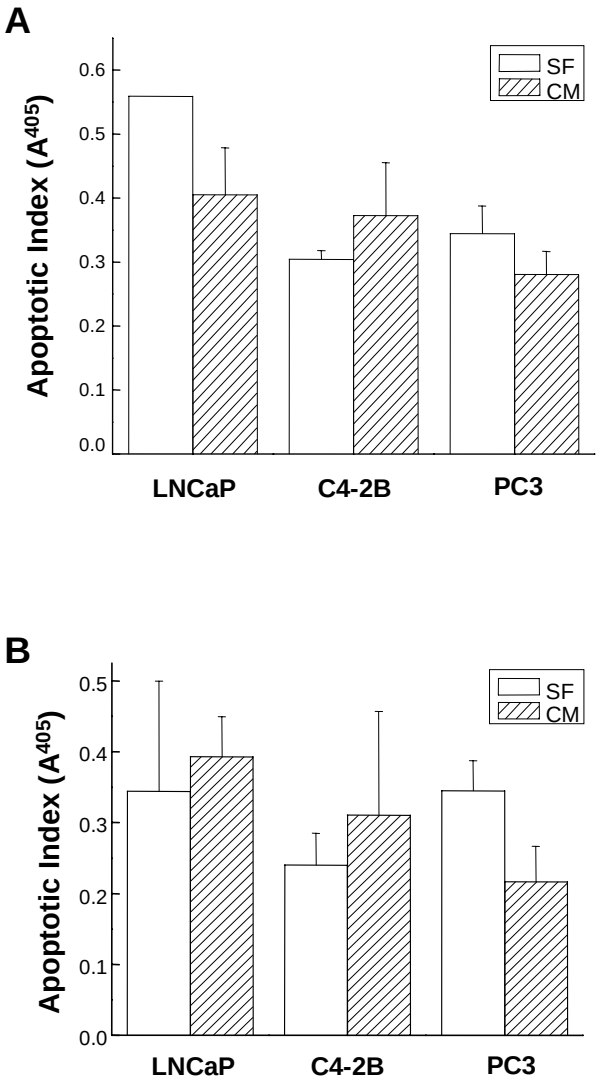

Figure 9. Conditioned medium harvested from other stromal cell lines failed to induce apoptosis in LNCaP and C4-2B cell lines. A. Cells were treated with foreskin cell line CM. B. Cells were treated with prostate stromal cell line CM. Fore skin cells and prostate stromal cells were seeded in T-75 until confluent. CM were harvested and filtered through 0.2nm filter. Prostate cancer cell lines were treated with CM for 2 days.

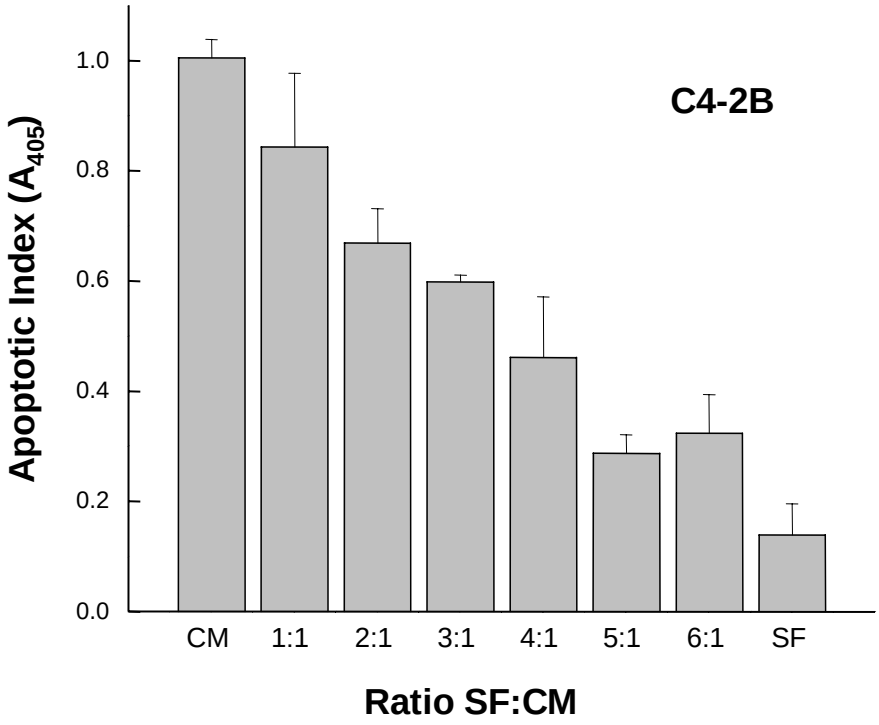

Figure 10. Apoptosis induced by conditioned medium (CM) was dose dependent. C4-2B cells were shown in the figure. Cells were treated with different ratio of SF:CM medium. DNA fragmentation ELISA assay was used to measure apoptosis.

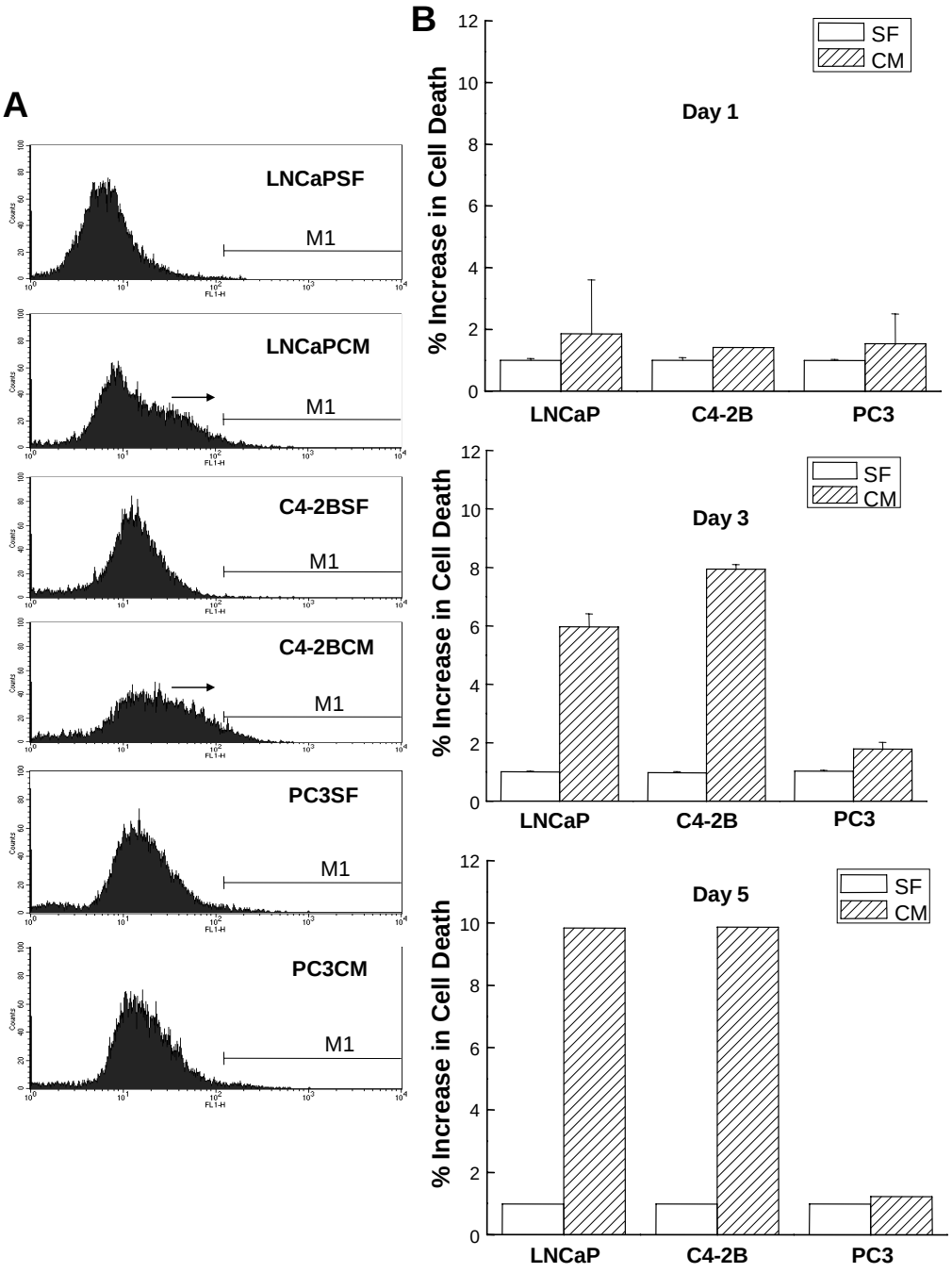

Figure 11. FACS assay showed increased apoptosis of LNCaP and C4-2B cells, but not of PC3 cells. A. FACS scatter grams showed increases of FAM-caspase 3&7 positive cells as "right" shifts [arrows]. Cells were grown in T-25 until confluent and then treated with CM or SF for 5 day time points (Only day 5 time points were shown here). The number of cells that shifted into the M1 region was counted and plotted as shown in B. **B. LNCaP and C4-2B cells showed increased cell death rates over 5 days in CM.** The percentage of SF induced cell death was standardized to 1.0 and CM increases in cell death were plotted.

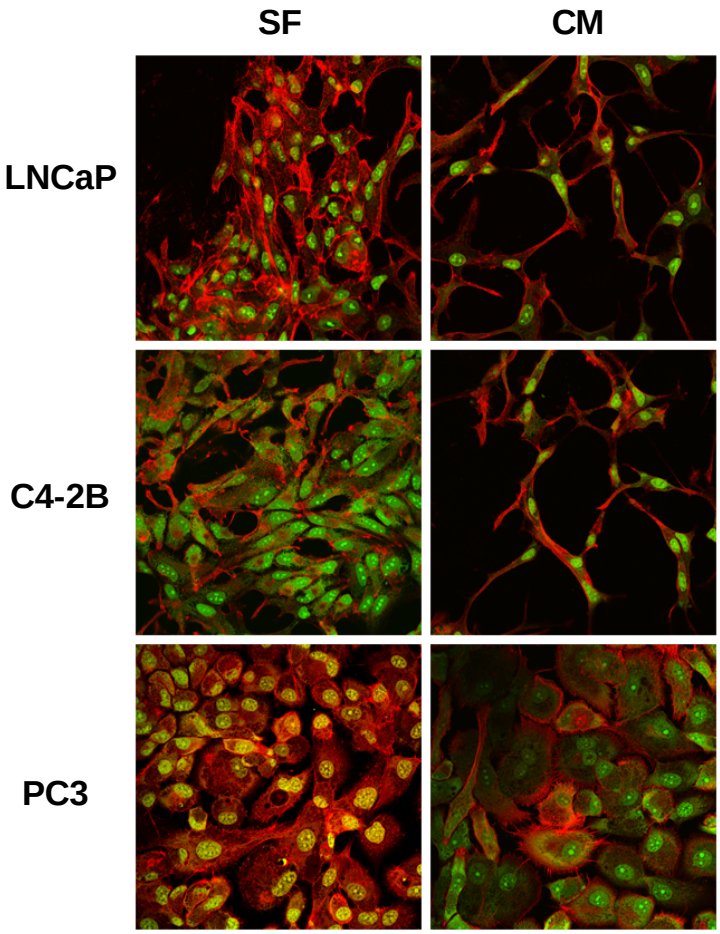

Figure 12. LNCaP and C4-2B cells, but not PC3, underwent morphological changes when grown in conditioned medium (CM) harvested from bone stromal cell lines. Cells were seeded in 8 well microscopic growth chambers and then treated with CM or SF medium for 5 days. Standard histoimmunochemistry assays were performed using Alexa Fluor Phalloidin for actin and SYTO13 for nuclei. Red: actin, Green: nuclear

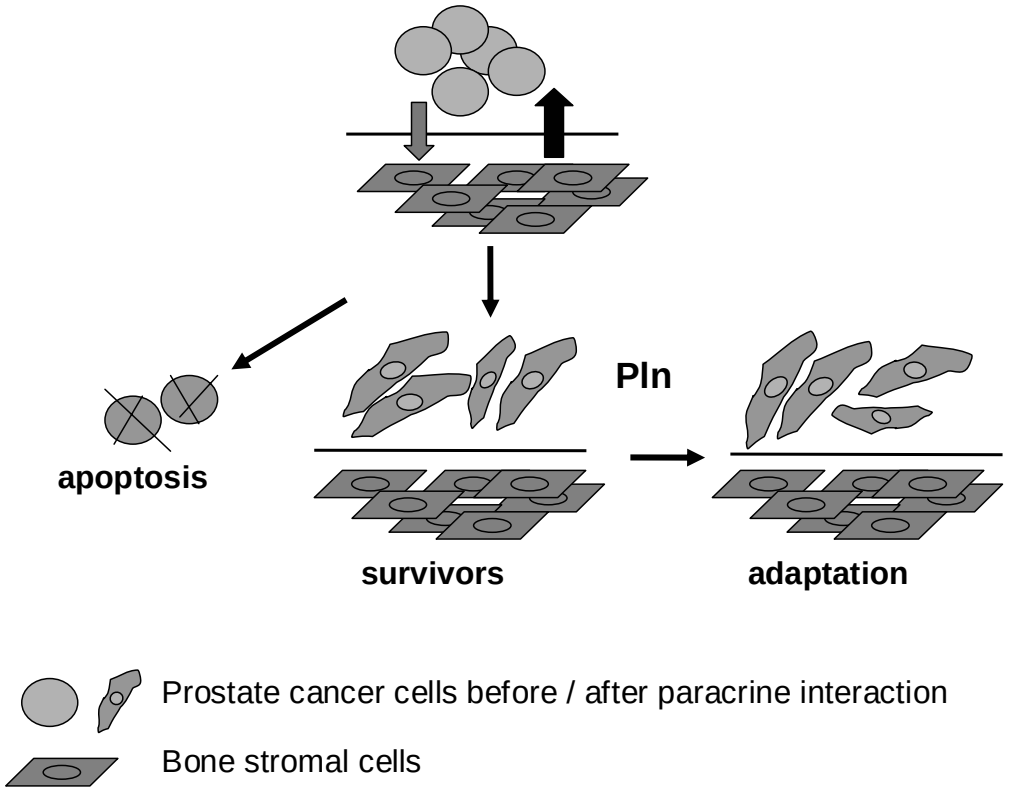

Figure 13. Model for paracrine interactions between bone stromal cells and prostate cancer cells in the bone microenvironment. PCa cells enter the bone environment and initiate paracrine cross talk with neighboring bone stromal cells. During the cross talk, some PCa cells are triggered to undergo apoptosis. Survivors undergo a series of genotypic and phenotypic changes and adapt to grow in the presence of bone stromal cells. This suggests an apoptosis and an adaptation stage exist during prostate cancer cell metastasis to bone. Pln plays an important role in the cell survival.

Key Research Accomplishment:

We have achieved many research accomplishments emanating from the training grant:

- **Manuscripts:**
  Zhang C., Chung L.W.K., Dodge G., Carson D.D. and Farach-Carson, M.C. (2006) *Function of PlnDI in Prostate Cancer Apoptosis and Survival.* Manuscript In preparation.
  Zhang C. Carson D.D. and Farach-Carson, M.C. (2006) *Paracrine Factors in the Bone Stromal Environment Induce Apoptosis in LNCaP and C4-2B Prostate Cancer Cells.* In preparation.

- **Abstracts:**
  Function of Perlecan in Prostate Cancer Cell Growth and Survival. The 8th International Conference on the Chemistry & Biology of Mineralized Tissues, October 17-22, 2004
  Function of Perlecan Domain I in Prostate Cancer Cell Survival. AACR Special Conference on Cancer, Proteases, and the Tumor Microenvironment. November 30-December 4, 2005
  Function of Paracrine Signaling in Prostate Cancer Cell Survival and Apoptosis in Bone Marrow Environment. The 97th AACR Annual Meeting, April 1-5, 2005

Reportable Outcomes

- Presentations
  Function of PlnDI in prostate cancer. Delaware Biotechnology Institute, April 2005
  Function of Paracrine Signaling in Prostate Cancer Cell Survival and Apoptosis in Bone Marrow Environment. Prostate Cancer Super-group Meeting, University of Delaware, May 2006
- Poster presentations at international and national conference (see abstracts)
- Two manuscripts in preparation for publication (see manuscripts)

Conclusions

During the two years supported by this Training Grant, we successfully finished and extended our proposed aims and are now writing these up for publication that will acknowledge the training grant. From Pln knockdown studies, we showed that Pln regulates PCa cell proliferation and PlnDI only is not sufficient for this function. We showed that Akt/MAPK pathways are not regulated directly by Pln during cell proliferation. We studied apoptosis of several PCa cell lines in response to apoptosis inducer camptothecin. We successfully rescued apoptosis of all PCa cell lines by Pln enriched CM from bone stromal cells. We also showed that PlnDI has the same rescue effect as Pln enriched CM, suggesting this maps to the HS bearing domain and growth factor delivery functions. We studied apoptosis of the Pln ribozyme knockdown clones induced by anti-Fas or camptothecin and compared to parentals, the knockdown clones showed more apoptosis. We showed that purified PlnDI protein can rescue the apoptosis

behavior of the knockdown clones to more closely resemble the parental cells that express normal levels of Pln. These experiments allowed us to assess the importance of PlnDI in PCa survival *in vitro*. Our follow up study is to further test if the GAGs or core protein are essential for the function of PlnDI by attempting rescue with HS or CS, alone or attached to BSA rather than Pln D1. We also will perform selective enzyme digestions with chondroitinase or heparanase to measure the relative contributions of the GAGs and the core protein. These systematic studies will dissect the role of PlnDI in controlling PCa apoptosis. In the closely related extended studies, we showed that there are natural factor(s) produced by bone marrow stromal cells that induce apoptosis in PCa cells. We hope to identify these factors in future work. Finally, we suggest that PlnDI protects PCa cells from apoptosis in the bone microenvironment, acting as a local survival factor as they further adapt to factors present in the bone environment. Targeting this feature may provide new mechanisms to selectively target these cells in metastatic disease.

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